The Genetic Basis of Pheromone Evolution in Moths

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Abstract

Moth sexual pheromones are widely studied as a fine-tuned system of intraspecific sexual communication that reinforces interspecific reproductive isolation. However, their evolution poses a dilemma: How can the female pheromone and male preference simultaneously change to create a new pattern of species-specific attraction? Solving this puzzle requires us to identify the genes underlying intraspecific variation in signals and responses and to understand the evolutionary mechanisms responsible for their interspecific divergence. Candidate gene approaches and functional analyses have yielded insights into large families of biosynthetic enzymes and pheromone receptors, although the factors controlling their expression remain largely unexplored. Intra- and interspecific crosses have provided tantalizing evidence of regulatory genes, although, to date, mapping resolution has been insufficient to identify them. Recent advances in high-throughput genome and transcriptome sequencing, together with established techniques, have great potential to help scientists identify the specific genetic changes underlying divergence and resolve the mystery of how moth sexual communication systems evolve.

INTRODUCTION

Traditionally, moth sexual pheromones have been studied as a static, fine-tuned system of intraspecific sexual communication that reinforces interspecific reproductive isolation. Most studies have focused on long-range attraction of males to female-emitted pheromones; the close-range attraction of females to male-emitted pheromones has received much less attention. The female signal is unimodal and exclusively chemical, produced in a well-defined gland, and readily quantified. The male behavioral response is robust and specific, and the well-studied pheromone sensory system serves as an important model for decoding olfactory preference in general. However, although female sex pheromones of >1,600 moth species have been enumerated (28, but see 98), the dynamic mechanisms of evolutionary change at work in pheromone systems are still obscure: How the female signal and male preference can concomitantly change remains a dilemma. To resolve this problem, researchers must identify the genes responsible for intraspecific variation in and interspecific divergence of the pheromone system. However, so far, only five such genes have been identified (2, 34, 36, 73, 75).

Recent developments in next-generation sequencing and functional genomics have provided new tools for extending classical genetic approaches to identify these genes and the molecular functions of the proteins they encode. Knowledge of the genes also permits sequence comparisons in a phylogenetic perspective to decipher evolutionary pathways (see 1, 2, 33, 34, 68, 72, 74, 81). Identifying sexual communication genes will also be of great interest from an applied perspective: Many moths are important agricultural pests, pheromones are used in pest control, and genes involved in pheromone production can be used to transform plants to produce pheromones (see 49). Here, we review existing data and new techniques to formulate a research paradigm for discovering the genes responsible for the intraspecific variation on which evolutionary divergence is based.

FEMALE SEX PHEROMONES

Basic Mechanisms of Moth Sex Pheromone Production

Female moth pheromones usually consist of a mixture of modified fatty acids of various lengths, which may have one or more double bonds in their hydrocarbon chains and terminal alcohol, aldehyde, or acetate ester functional groups (type I pheromones, reviewed in 62, 111, 129). Some key enzymes in pheromone production have been well studied, and one mechanism of hormonal control has been established; we summarize the main findings below.

Control of sex pheromone production. In many moth species, female sex pheromone production is regulated by the nightly release of pheromone biosynthesis activating neuropeptide (PBAN) from the subesophageal ganglion (62, 111, 112). PBAN activates a G-protein–coupled receptor, PBANr, in the pheromone gland (57, 66). When PBAN binds to PBANr, a calcium channel opens and the influx of Ca^{2+} initiates pheromone production via second messengers (110) (see **Figure 1**). In *Heliothis virescens*, activated acetyl coenzyme A (CoA) carboxylase stimulates de novo synthesis of fatty acids (29). In *Bombyx mori*, previously synthesized fatty acids are stored as triglycerides in lipid droplets (94, 95) and mobilized by phosphorylation of a lipid storage droplet protein (104). PBAN may also stimulate the last step of fatty acyl reduction (5). The mechanism of PBAN activation is best understood for *B. mori*, in which researchers have used RNA interference (RNAi) to disrupt PBANr and several downstream proteins (56, 105). However, different mechanisms probably occur in other species (131): In *Trichoplusia ni*, for example, pheromone is continuously produced and is not regulated by PBAN (127). Thus, considerable diversity exists in the control of pheromone production, and how this diversity is generated through evolution remains unexplored.

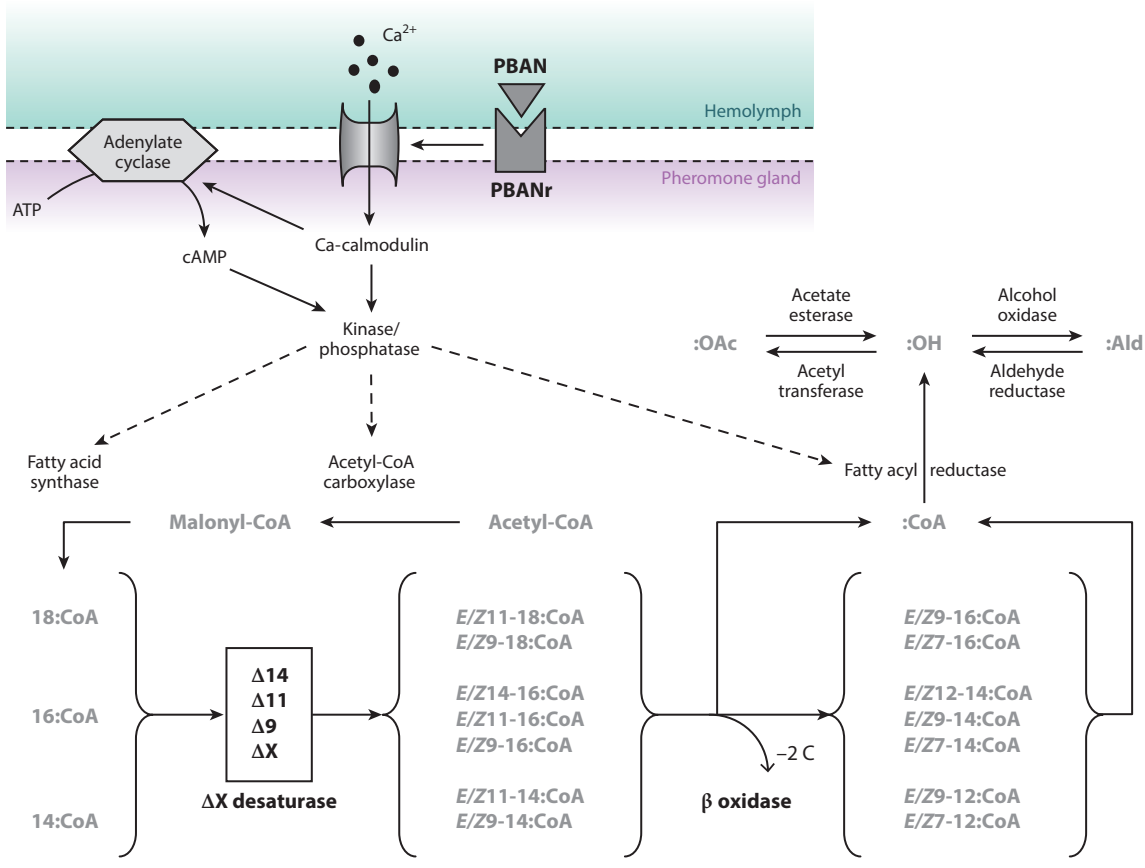

Figure 1

Schematic drawing of the possible sex pheromone biosynthetic pathways in female moths. When pheromone biosynthesis activating neuropeptide (PBAN), produced in the subesophageal ganglion, binds to the PBAN receptor (PBANr) in the membrane of the sex pheromone gland, different enzymes in the fatty acid pathway are activated, depending on the species. Intermediate products (*in brackets*) may be stored as di- and triacylglycerides or directly converted to their end products, alcohols (OH), aldehydes (Ald), or acetate esters (OAc). Some compounds can be produced via multiple pathways; for example, *Z*9-16:CoA can be produced via Δ11 desaturation of stearic acid (18:CoA) with subsequent β-oxidation or via Δ9 desaturation of palmitic acid (16:CoA). Abbreviation: -2 C, chain shortening of two carbon atoms.

Sex pheromone biosynthesis. Detailed biochemical studies conducted in the 1970s and 1980s (12) revealed a list of enzymatic activities required to synthesize fatty acids and convert them to di- and/or triacylglycerides for storage and subsequent modification (reviewed in 62, 129) (see **Figure 1**). Enzymes catalyzing later modifications include desaturases, which introduce one or more double bonds to carbon chains, and fatty acyl reductases (FARs), which convert CoA thioesters to alcohols. Alcohols can be pheromone components themselves, or alcohol oxidase can convert them to aldehydes (aldehyde reductase can convert aldehydes to alcohols). Alcohols can also be converted to acetate esters by acetyl transferase, and acetates can be converted (back) to alcohols by acetate esterase. All these enzymes are members of large protein families, making it challenging to identify the proteins responsible for determining species-specific pheromone blends.

Genetic Analyses of Sex Pheromone Variation

A general approach to dissecting pheromone biosynthetic pathways starts with identifying large families of candidate genes by analyzing the sequence of the transcriptome and/or the genome of a species of interest. Then, the scope is narrowed using evidence such as tissue specificity, species specificity, or genetic linkage data. This approach rarely results in a single candidate, but it can be useful in shortening the list of genes to be subjected to further functional analysis. The best-studied gene families involved in pheromone synthesis are desaturases and FARs.

Desaturases. Desaturases are integral membrane proteins that occur ubiquitously in eukaryotic cells, where they play a primary role in the homeostatic regulation of membrane lipids' physical properties in response to cold (68). The first moth desaturases were cloned and functionally characterized from complementary DNA (cDNA) isolated from the pheromone glands of *T. ni* (67) and *Helicoverpa zea* (118). Knipple et al. (68) conducted a phylogenetic analysis of all identified desaturases in eight moth species and found six main desaturase subfamilies, which are also present in *Drosophila melanogaster*. This desaturase expansion occurred before the split of Diptera and Lepidoptera (confirmed in 81); the Δ9 desaturase lineages with NPVE and KPSE domains are highly conserved, whereas the Δ11 desaturase lineage with the xxxQ domain is more divergent (68).

Roelofs et al. (117) have suggested that pheromone divergence in the genus *Ostrinia* results primarily from desaturases. A double bond is introduced in the acyl chain only at the 11 position in *O. nubilalis* and only at the 14 position in *O. furnacalis*, although Δ11 and Δ14 desaturase transcripts are found in the glands of both species. Functional analysis has shown that Δ11 and Δ14 desaturases from both species are catalytically competent, so the species' pheromone differences cannot be explained by a reciprocal loss of function. Additional desaturase genes (*ezi*-Δ11-α, *ezi*-Δ11-β) have been identified in both species, pointing to the complex evolution of this enzyme family, but their role in pheromone biosynthesis has not been established (140). The authors suggest that a novel pheromone may result from activation of a latent enzyme already encoded in the genome rather than evolution of a new enzyme.

Likewise, in the pheromone glands of both *O. furnacalis* and *O. scapulalis* (the latter of which has a double bond only in the 11 position of the acyl chain, like *O. nubilalis*), Sakai et al. (119) found transcripts of both Δ11 and Δ14 desaturases. However, Δ11 transcripts were very rare in *O. furnacalis* and Δ14 transcripts were very rare in *O. scapulalis*; specific antibodies showed the same pattern for the desaturase proteins. Interspecific hybrids had high levels of both transcripts, expressed both proteins, and produced both types of desaturated products. Thus, the authors suggest the existence of a transcriptional repressor with two recessive alleles: The *di14* allele in *O. scapulalis* suppresses Δ14, and the *di11* allele in *O. furnacalis* suppresses Δ11 (119).

The primitive species *O. latipennis* produces only a 14-carbon alcohol with a *trans* double bond in the 11 position of the acyl chain (33). The pheromone gland–specific desaturase identified in this species has only 55% amino acid identity with the active Δ11 desaturases from other *Ostrinia* species and is more similar to *ezi*-Δ11-α (140). Expression of this desaturase in the pheromone gland may thus be the ancestral condition in *Ostrinia*; as other desaturases capable of inserting *cis* or *trans* double bonds in the 11 or 14 position of the acyl chain became expressed in other species, generating a more diverse pheromone blend (33), the expression of the ancestral desaturase may have become suppressed.

In *B. mori*, researchers identified the desaturase responsible for the two double bonds in the pheromone bombykol by cloning and functionally expressing all desaturases expressed in the moth's pheromone gland (101). This enzyme (Bmpgdesat1) catalyzes both consecutive desaturation steps in bombykol biosynthesis. Targeted disruption of Bmpgdesat1 by RNAi greatly reduces

the production of bombykol (105). Desaturases with dual catalytic activity have also been identified in *Spodoptera littoralis* (115), *T. ni* (122), and *Dendrolimus punctatus* (80) and seem to represent a new family of desaturases in Lepidoptera.

A comprehensive survey in two endemic New Zealand leafroller moth genera, *Ctenopseustis* and *Planotortrix*, yielded one Δ10 and two Δ9 pheromone-synthesizing desaturases, as well as a Δ6 and a terminal desaturase (1). Sibling species of each genus had nearly identical desaturases but differed in Δ10 desaturase mRNA levels, corresponding to the presence of a pheromone component produced by Δ10 desaturation of the precursor. Crosses of the two *Planotortrix* species produced evidence of a *trans*-acting transcriptional repressor and revealed a *cis*-acting regulatory mutation in an activator-binding site upstream of the Δ10 desaturase (2). As in *Ostrinia*, a defined set of pheromone components is produced by expressing only a subset of the desaturase genes present in the genome; this points to the importance of regulatory changes in causing species differences.

Fatty acyl reductases. The first pheromone gland–specific FAR (pgFAR) was identified in *B. mori*. It showed a strong substrate preference for the bombykol precursor (*E*,*Z*)-10,12–16:acid compared with other compounds [(*E*,*E*)-10,12–16:acid, *Z*11–16:acid)] when heterologously expressed in yeast (102). Its key role was confirmed by RNAi, which greatly reduced the production of bombykol in vivo (105).

By combining a candidate gene approach with genetic mapping (see below), Lassance et al. (73) identified *pgFAR* as the gene underlying pheromone variation among the E and Z strains of *O. nubilalis*. Both strains produce the same ratio of E and Z pheromone precursors, but the E-strain enzyme preferentially reduces the E isomer and the Z-strain enzyme prefers the Z isomer. These enzymes are encoded by alternate alleles at the same locus (73). Phylogenetic analysis of *pgFAR* in eight *Ostrinia* species showed strong discordance between the gene and species trees (74). A shift in topological position was found across numerous sequences, possibly owing to selection. Subsequent mutagenesis experiments showed that a single amino acid substitution in the C-terminal region can alter the ability of the protein to catalyze the reduction of Z9-14 and Z12-14 acyl precursors, which is the main difference between the *O. nubilalis* and *O. furnacalis* pgFAR variants (74).

Liénard et al. (79) sequenced and functionally characterized the pgFARs of three closely related *Yponomeuta* species that produce different mixtures of C14- and C16-fatty alcohols and their derivatives (91). The *Yponomeuta* pgFAR orthologs showed similar biochemical activities, all accepting a broad range of substrates, suggesting that their function in the pheromone gland evolved in the genus before species diversification. Hence, evolutionary divergence probably occurred upstream in the biosynthetic pathway, possibly in the desaturases; in this case, the pheromones produced depend on the fatty acyl precursors available and not on any difference in pgFAR activity (79).

Acyl-CoA binding proteins. A pheromone gland–specific acyl-CoA binding protein (pgACBP) has been isolated from *B. mori* (96). ACBPs bind to straight-chain acyl-CoA esters (C14–22) with high affinity, protecting them from hydrolysis. The researchers who isolated the protein speculated that it could function as a carrier of the acyl-CoA esters used in pheromone biosynthesis, and they showed that RNAi prevents triglyceride accumulation in lipid droplets and thus reduces the availability of the bombykol precursors (95).

Transcriptome analysis. Several research groups have analyzed the transcriptome of active female sex pheromone glands, either by extracting RNA from glands in the scotophase or by injecting PBAN to activate pheromone biosynthesis (45, 61, 124, 135, 144). Their studies have generated lists of additional pheromone-related candidate genes based on the enzyme activities

that were biochemically identified in the 1970s and 1980s (see **Figure 1**). Under the premise that variation in expression underlies differences in pheromone blends, a few studies have assessed variation in gene expression levels using microarrays (17, 125).

Genetic linkage analysis. When two reproductively compatible populations have different pheromones, the underlying genetic basis can be investigated by crossing the populations and analyzing the progeny. In Lepidoptera, the female is the heterogametic sex (ZW or ZO, in contrast to the homogametic ZZ males), and no crossing-over occurs in females, making linkage within chromosomes easy to detect (54). When genetic markers are added to the analysis, chromosomal effects can be detected using QTL (quantitative trait locus) analysis (87) and candidate genes can be tested by mapping them to the chromosomes. This usually results in the rejection of most candidates, but in two cases, crosses have led to the genes responsible for population differences, as described above (2, 73).

Most genetic analyses have been conducted by phenotyping F_1, F_2, and/or backcross females to infer whether pheromone variation is sex-linked or autosomally inherited and whether one or more genes are involved (see **Supplemental Table 1**; follow the **Supplemental Material link** from the Annual Reviews home page at **http://www.annualreviews.org**). Most studies have not employed genetic markers and have found inheritance patterns that could be explained by one or two major autosomal genes. The addition of genetic markers to such studies enables greater resolution. Interspecific crosses of the closely related *H. virescens* and *H. subflexa* have revealed several autosomal QTLs affecting the abundance of different unsaturated pheromone components and the presence of acetate esters (39, 123). Intraspecific crosses between *H. subflexa* populations differing in acetate ester amounts, and between *H. virescens* populations differing in the amount of a key unsaturated component, have produced QTLs that overlap previously detected interspecific QTLs (41, 42). This suggests a continuum between intraspecific and interspecific pheromone variation.

Supplemental Material

MALE RESPONSE

Male response to and preference for female sex pheromone are dictated by the olfactory circuitry, as reviewed by Galizia & Rössler (35) and briefly outlined here. Pheromones are detected by olfactory sensory neurons (OSNs) housed in hair-like sensilla on the antenna. Hydrophobic pheromones pass through pores in the sensillar cuticle, bind to odorant binding proteins (OBPs), and are carried through the aqueous lymph to OSN dendrites. Dendrites express pheromone receptors (PRs), which, on binding the OBP-pheromone complex, induce ion channels to open, depolarize membranes, and generate action potentials. The PR largely determines OSN specificity, whereas OBPs modulate this specificity. OSNs can be classified into different subtypes depending on their spike amplitude and the sensillum subtype in which they are expressed. All OSNs of the same class send axonal projections to a single glomerulus in the antennal lobe of the moth brain. From glomeruli, projection neurons send information to the lateral horn, a brain area implicated in assessing the valence of a stimulus. From here, axonal projections are sent to motor control centers. Because of the experimental accessibility of the antenna, most studies of variation in male preference have focused on the peripheral sensory system housed there.

Neural Correlates of Male Preference

In Ditrysia moths, PRs are a monophyletic clade of olfactory receptors devoted to pheromone detection (69, 70, 99, 120). Expression of PRs is restricted to sensilla trichoidea, indicating a phylogenetic or functional constraint intimately tied to other trichoid factors, including sensillum ultrastructure, OBPs, sensory neuron membrane proteins, or chemosensory proteins (10, 59, 92, 145).

PR protein structure dictates binding specificity and is particularly critical in qualitative pheromone preference shifts. For example, all *Ostrinia* species examined have seven functional PRs (99). However, in *O. furnacalis* the affinity of one receptor has shifted from Δ11 acetates to Δ12 acetates, which corresponds to a shift in pheromone production and preference from Δ11 to Δ12 compounds (75).

More often, however, interspecific differences in pheromone preference are linked to a change in OSN identity [as observed in *O. nubilalis* (90, 116), *Ctenopseustis* spp. (32), *H. subflexa* and *H. virescens* (76), and *B. mori* (34)] and/or a change in the ratio of different pheromone-sensitive sensillum trichodea subtypes [as observed in *Agrotis* spp. (53), *H. virescens*, *H. subflexa* (7, 76, 139), and *Helicoverpa* spp. (11)]. In species with only one pheromone sensillum type, a shift in the preferred ratio of the binary blend was correlated with swapping of the neuronal identity of the cohoused OSNs, each expressing a different PR tuned to one of the components [as observed in *Ctenopseustis* spp. (51) and *O. nubilalis* pheromone strains (52)]. Interestingly, in *O. nubilalis*, male E and Z strain hybrids prefer a more equal ratio of E and Z isomers than the parental strains do, and the two glomeruli are roughly equal in size (63, 64). This indicates that the relative size of sensory neurons (as reflected by spike amplitude differences; see 69) translates into the relative volume of glomeruli, which is linked to preference. In drosophilids, glomerular volume is also linked to pheromone preference (23), as well as preference for food odors (58).

Two additional factors have been linked to male preference: the relative sensitivity of OSNs and tuning breadth. For example, *T. ni* males selected for attraction to a novel female blend (88) exhibited lower OSN sensitivity to the overexpressed compound, *Z*9-14:Ald (24). Similarly, compared with their normal counterparts, the 3–5% of *O. nubilalis* males attracted to *O. furnacalis* pheromone (83, 84, 117) show subtle differences in the tuning breadth of their underlying neurons (25). Conversely, compared with their normal counterparts, neurons in the rare *O. furnacalis* males attracted to *O. nubilalis* pheromone do not respond to a compound that inhibits attraction of most other *O. furnacalis* males (26). A broadened behavioral acceptance of blend variation may also arise from the inability of the olfactory circuitry to distinguish differences: The six compounds in the pheromone blend of *T. ni* appear to be partially redundant, as blends with altered ratios or missing components produce the same behavioral attraction (82) and neurophysiological responses (130). This allows for variation in female-produced ratios, even of behaviorally important components, without the need for a concomitant change in male response.

Other factors also alter the sensitivity and tuning breadth of OSNs, including PR coexpression and OBPs. In *O. nubilalis*, OSNs tuned to the behavioral antagonist coexpress at least five different PRs in a mosaic fashion (69). Accordingly, these OSNs are broadly tuned to a large number of heterospecific compounds (69). Changes in PR coexpression affect OSN tuning breadth and behavioral response. Also, OBPs are stereotypically expressed in subsets of OSNs, and they fine-tune the sensitivity and specificity of PRs in vitro (31, 44, 46, 121), which implies that a change in the tuning breadth of OSNs could result from changes in the expression or binding affinity of OBPs.

More central processes have also been implicated in shifts in pheromone preference (7, 36), such as antennal lobe glomeruli targeted by pheromone-sensitive neurons (11, 63, 76). In heliothine moths, glomerular position and specificity differ between species with different preferences (11, 76, 139). Whereas such changes may correlate with shifts in preference, they do not currently help explain them, because the consequences of topological shifts in glomeruli are unknown. In *D. melanogaster*, misexpression of Dscam, a protein involved in axonal guidance, causes topological rearrangements of the dendrites of antennal lobe projection neurons. However, OSN axons match the mistargeting, such that the pairing between OSNs and projection neurons remains the same (146). Similarly, in hybrids of *O. nubilalis* E and Z strains the functional topology is identical to that of the E strain, even though hybrid males show a preference intermediate between the two

parental strains (64). Thus, the precise relationship between OSN/glomerular connectivity and pheromone preference is still poorly understood.

Genetic Analyses of Male Preference

Supplemental Material

Genetic studies of male preference include investigations of heritable variation in preference (18), selection experiments (e.g., 4, 88), crossing experiments (20, 50, 64, 106, 116), and a few QTL studies (27, 36) (see **Supplemental Table 1**). Early studies using hybridization and backcrosses demonstrated that the difference in physiological response and pheromone preference between the E and Z strains of *O. nubilalis* was sex-linked (20, 52, 64, 69, 90, 106, 116). QTL mapping studies confirmed that the preference locus is on the Z chromosome (27), but it mapped to a different position than the tightly linked cluster of genes encoding PRs, including the E11 and Z11 PRs (72, 142). This was surprising, as preference correlated with a swap in OSN identity across the three neurons coinhabiting the same trichoid sensillum (69). Thus, the gene responsible for the difference in ratio preference and how it affects peripheral factors are still under investigation.

Pheromone communication in heliothine moth species appears to rely on a limited set of type compounds. Which compounds act as pheromone or as antagonist differs between species. For example, the pheromone blend of *H. subflexa* includes *Z*11-16:OAc, unlike that of *H. virescens*, which acts as a behavioral antagonist for *H. virescens* (40, 134). Behavioral, sensory, physiological, and QTL analyses of hybrids and backcrosses between the two species (7, 36, 133) indicate that the main QTL for preference is tightly linked to a cluster of PRs (HR14, HR15, and HR16) tuned to *Z*11-16:OAc (HR14) and *Z*11-16:OH (HR16) (36, 136). Thus, unlike in *Ostrinia*, variation in expression of PRs in *Heliothis* is more directly responsible for preference differences; however, tight linkage prevents recombination within the PR cluster and a final determination of whether a single gene is responsible for preference.

In *B. mori*, mutations in the transcription factor gene *Bmacj6* reverse male preference from bombykol to bombykal (34). Mutants' antennae have the same levels of the bombykal-tuned PR BmOr3 but drastically reduced levels of the bombykol-responsive BmOr1. Moreover, the large toroid glomerulus that responds to bombykol in wild-type *B. mori* responds to bombykal instead in the mutant strain. Interestingly, the homologous gene *acj6* in *D. melanogaster* is involved in OSN identity in that species (113). This suggests that *Bmacj6* is involved in specifying sensory neuronal identity in *Bombyx*, by affecting PR expression, glomerular targeting, or both.

Although the proximate causes of shifts in preference are still obscure, the neural correlates mentioned above strongly suggest a role of transcription factors and their binding motifs. Studies in *Drosophila* have demonstrated that the combination of binding motifs in the regulatory domain of olfactory receptors determines the neuron in which they are expressed (30, 113). A combinatorial code of transcription factors also appears to be involved in regulating the ratio of sensillum types and subtypes. The transcription factors atonal, amos, and lozenge regulate choice among major sensillum types in a combinatorial fashion (37, 47, 48), whereas the choice of trichoid subtypes involves the transcription factor rothund (78). Interestingly, Koutroumpa et al. (69) have noted that the trichoid sensillum tuned to pheromone in *O. nubilalis* expresses receptors in a way that suggests an ongoing split of this sensillum into two subtypes: The lateral subtype expresses a receptor that is not expressed in the medial subtype.

NEW DEVELOPMENTS IN GENETIC ANALYSES

Why have so few genes been identified in a system in which the phenotypes (the female pheromone signal and the male response) are so well defined and described? The genes for many biosynthetic enzymes involved in the female signal remain to be cloned, and the transcription factors that

regulate their expression and thereby alter pheromone blends are unknown. As for male response, several PRs are well characterized, as described above, but also in these cases the transcription factors that regulate their expression in specific sensilla and dictate the proper synaptic connections of sensory neurons are unknown. Recent technological advances can fill the gaps, however, as described below.

Genome sequencing has advanced considerably, and the genomes of several species of Culicidae, Lepidoptera, and Coleoptera have been completed (21, 103, 114). This progress has been enabled by technologies (454, Illumina) that can produce hundreds of millions of short sequencing reads and by computational advances in assembling the overlapping reads into contigs up to hundreds of kilobases. These assemblies are often highly fragmented, however, owing to intraspecific polymorphism and the presence of interspersed, highly repeated sequences, such as transposable elements. Even fragmented assemblies can produce a reasonably complete list of protein-coding genes, which is very useful in initiating the candidate gene approach in a new organism. Comparing the genomes of closely related species or populations has yielded insights into variation in some conspicuous traits, such as wing color patterns (71). Often, linkage information from the physical map and genome sequence of one species, such as the domesticated silkworm *B. mori*, aids in interpreting genetic linkage information from related species owing to partial conservation of chromosomal structure and gene content (42). An existing genome assembly can be used to support sequence comparison among individuals or populations, as scientists can resequence comparison genomes at low coverage and align the reads to the existing genome to detect polymorphisms. This approach was recently used to gain insight into the genetic basis of wing color and migratory tendencies among worldwide samples of the monarch butterfly (*Danaus plexippus*) (143). Similar genome-wide association studies could be used to hone in on differences in recently diverged pheromone races or species.

Transcriptome sequencing, which provides a useful snapshot of protein-coding genes that are expressed in specific sexes or tissues, has also benefited from technological advances. High-throughput RNA-seq methods not only provide much greater coverage than microarrays do, allowing rare but important transcripts to be identified, they can also be used to estimate relative expression levels more accurately. Targeted to the female pheromone gland (124, 135) or the male antenna (43, 77), transcriptome sequencing has identified large gene families involved in pheromone biosynthesis, detection, and degradation. However, this information is still not sufficient to determine which particular members of these gene families are most important to biological function.

Genetic marker technologies furnish the link for identifying associations between sexual communication traits and locations in the genome. The focus of these approaches has evolved from low-density markers, such as RFLPs (restriction fragment length polymorphisms), to high-density markers, such as RAPDs (random amplified polymorphic DNA) and AFLPs (amplified fragment length polymorphisms), which are considered anonymous because only the mapping information and not sequence information is (initially) known, to ultrahigh-density markers, such as RAD (restriction site–associated DNA) tags (6) and GBS (genotyping-by-sequencing) markers (109), for which DNA sequence information accompanies the mapping information. It is now feasible to produce high-resolution linkage maps with sequence-based markers that can be matched to an existing genome sequence for a species or compared to the genome sequence of a related species (9). With sufficient effort, the positional cloning approaches that have identified mutations involved in pheromone signaling in *B. mori* (34) can now be applied in other Lepidoptera. Phylogenetic analysis of gene families can provide evidence of selection-driven accelerated change in specific family members, which may point to a change in function in pheromone production or detection (141). This approach was useful in *Ostrinia* for identifying the FAR involved in pheromone biosynthesis (73) and in homing in on odorant receptors important in pheromone preference (72, 75).

RNAi offers one way to test the effect of a gene on a phenotype by specifically downregulating the gene's expression. When double-stranded RNA (dsRNA) with sequence identity to a given gene enters a cell, it is cleaved into short 21-mers, which destroy mRNA of the corresponding gene (138). Although successful in *B. mori* (95), RNAi is problematic in many other Lepidoptera (128), primarily because active dsRNases in the midgut and hemolymph rapidly destroy dsRNA and not all cells readily take up dsRNA. Plant-mediated RNAi, in which larvae consume genetically modified plants that express dsRNA targeting an insect gene, has been successful in some species (8, 93).

Genome editing using the recently designed CRISPR/Cas9 system is a highly promising technology for disrupting genes in nearly any organism (16). By injecting embryos with a plasmid construct expressing the Cas9 nuclease and a gene-specific guide RNA, a targeted gene may be mutated in the germline and a stable knockout strain of the organism produced. Its ability to create knockouts has recently been demonstrated for *B. mori* (137). Modifications of the technology to replace an existing gene with a different functional gene sequence have been developed but not yet validated in Lepidoptera. Transgenic approaches are widely employed in *B. mori*, but they remain out of reach for most other Lepidoptera. However, researchers are increasingly transforming *Drosophila* with lepidopteran genes in functional analyses, especially of PRs (100, 132).

EVOLUTIONARY PERSPECTIVE

In the moth species studied so far, the genomic regions involved in female pheromone production and male response do not appear to overlap: Variation in the female pheromone is generally autosomally controlled, whereas the male response is either sex-linked (in *Bombyx* and *Ctenopseustis*), autosomally controlled by genes on linkage groups other than those that control the female pheromone (in *Heliothis*), or both (in *Ostrinia*) (32, 34, 36, 51, 64, 90, 106, 116). This makes Fisherian runaway selection unlikely; without a genetic association between signal and response, genetic changes evolve independently. Instead, the evolutionary scenarios described below have been proposed for moth sexual communication. Until now, these have been posed and evaluated with respect to phenotypes of sexual communication, in the absence of knowledge of most of the genes underlying these phenotypes. When these genes are eventually known, more powerful tests based on comparisons of allele frequencies among populations and over time will be possible.

Stabilizing Selection

Sexual communication in moths is generally considered to be under stabilizing selection: Variation in the female signal is limited, and males typically prefer the most common signal (20, 86, 147). Also, the predominant role of moth sexual communication is species recognition, as closely related species have similar mating signals and thus run the risk of interspecific attraction (14, 89, 107). However, stabilizing selection cannot explain how sexual communication systems evolve or the diversity of moth pheromone blends (60, 89, 126).

Directional Selection

When two closely related species with similar communication channels occur in sympatry, communication interference can cause reproductive character displacement, which has been hypothesized to counter stabilizing selection (13, 15, 38, 91, 97). In areas where *Hemileuca electra* and *H. burnsi* are sympatric, mate recognition signals diverge more than in areas where they are allopatric (97), and the same is true of areas where *Lymantria monacha* is sympatric with *L. fumida* in Japan (38). When the closely related moth species *H. virescens* and *H. subflexa* are sympatric,

H. subflexa females produce a relatively higher amount (>5%) of acetate esters that prevent communication interference, causing directional selection (40).

The Wallflower Effect

The basic assumption of the wallflower effect is that females mate less frequently than males. In this model, males eventually mate with less attractive, variant females producing a suboptimal pheromone blend (wallflowers), because the relative frequency of such females increases over the course of the mating season as the availability of more attractive virgin females decreases (22). Eventually, even these variant females mate and contribute to the next generation. Thus, in theory, the wallflower effect maintains heritable variation in pheromone production in spite of male-imposed stabilizing selection.

Asymmetric Tracking Hypothesis

The asymmetric tracking model assumes that male preference varies more than the female pheromone does because of asymmetrical parental investment (108). A broad male preference function maximizes the male's ability to locate potential partners; this increases his fitness because every mating increases the number of offspring he sires. Such a broad preference reduces the selection pressure on the female pheromone. This suggests that male preference functions may track variation in the female pheromone instead of constraining it.

Thus far, experimental findings confirm neither the wallflower model nor the asymmetric tracking model, as male preference is generally fine-tuned to the species-specific pheromone blend, as described above. However, although tuned to the female blend, the specificity of male preference may be overemphasized. Generally, males accept a range of blends. For example, using naturally occurring variants of the female almond moth (*Cadra cautella*) pheromone in a wind tunnel assay, Allison & Cardé (3) found unimodal preference functions only when two odor mixtures were offered; no-choice tests resulted in a flat preference function. In addition, Hemmann et al. (55) found a possible trade-off between male sensitivity and breadth of response; *T. ni* mutant males with broader response profiles exhibited lower sensitivity than wild-type males, whereas hybrid males exhibited both a narrow response profile and reduced sensitivity, suggesting a hybrid disadvantage. These findings suggest that males possess two response traits, sensitivity and breadth of response, both of which should be considered in evolutionary scenarios.

Rare Male Hypothesis

This hypothesis was prompted by the discovery of Roelofs et al. (117) of cross-attraction between the closely related species *O. nubilalis* and *O. furnacalis*. Whereas *O. nubilalis* uses a blend of Δ11 acetates, *O. furnacalis* uses Δ12 acetates. Cross-attraction is uncommon. However, 3–5% of *O. nubilalis* males were cross-attracted to the heterospecific blend, suggesting the presence of a factor that made them more broadly tuned (83, 84, 117). Rare, broadly responding males may thus form a bridge of relaxed selection between two optima in a disjunct pheromone landscape.

Male Mating Mistakes

An additional complicating factor is that the fidelity of male preference varies during flight to the female. For example, in the Oriental fruit moth (*Grapholita molesta*), overall male response specificity is predominately controlled by the early stages of upwind flight and upwind flight

arrestment (85). Similarly, *O. nubilalis* males continue to fly upwind to a pheromone odor source even if the odor has been switched midflight to that of the other pheromone strain (65). Such male mating mistakes should mitigate stabilizing selection and allow for the existence of variant females producing a nonattractive pheromone blend, albeit at low frequency. Similarly, *T. ni* males pre-exposed to or flying in a background cloud of behavioral antagonist are less affected by the compound during orientation (88). Even species that are reportedly highly selective may be less so in choice situations in a wind tunnel (19, 65, but see 3). Thus, phenotypic plasticity in male preference may form nonadaptive valleys connecting pheromone optima (55, 65), in spite of the selectivity of male preference.

CONCLUSIONS

Although our understanding of the genetic basis and evolution of moth sexual communication has progressed, we are still far from drafting clear scenarios for the evolution of pheromones, even for the moth species studied most thoroughly, *O. nubilalis*. A clear understanding will only become possible when we identify both the genes underlying the female signal and the genes underlying male preference. Male response and preference seem to be especially complicated, consisting of different subcomponents (sensitivity and breadth of response) and involving response profiles that depend on choice or no-choice situations and variation in selectivity during flight. Many candidate genes exist for both signal and response, but, as they generally belong to large gene families, identifying the specific gene(s) responsible for the initial changes causing divergence in communication channels remains challenging. By combining different approaches, including genetic linkage analysis, mapping candidate genes onto genetic maps, and functional analyses using RNAi and/or genome editing, such identification will become possible. A combinatorial approach is needed because not only structural genes but also regulatory elements, such as transcription factors and activators or repressor binding sites, may underlie the initial divergence in both signals and responses. Now that sequence-based markers and genomes have become available, genotyping will become much easier, which should result in the identification of many more specific genetic changes underlying sex pheromone diversification in moths in the near future.

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